The Expression of a Functional, Secreted Human Lysyl Hydroxylase in a Baculovirus System

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This study reports the expression of functional human lysyl hydroxylase (LH), a post-translational modifying enzyme that catalyzes the hydroxylation of the lysine residues essential for cross-linking in collagen biosynthesis. We have developed a novel baculovirus system for the expression of LH, a protein that exists normally within the lumen of the endoplasmic reticulum, using a powerful baculovirus signal sequence for secretion. The supernatant from Sf9 cells infected with the viral recombinant showed significant LH activity that increased linearly with supernatant concentration, whereas there was no detectable LH activity in the cell pellet. Silver staining of the fractions purified from the active supernatant by concanavalin A Sepharose chromatography and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis demonstrated an 85-kDa protein (the expected size of the LH subunit) that was most prominent in those fractions with the highest LH activity. N-terminal amino acid sequencing verified that the N-terminal primary structure of this 85-kDa protein was identical to human LH. Moreover, the activity of the expressed protein was shown to be dependent on the presence of Fe^{++}, ascorbate, and α-ketoglutarate, three essential cofactors for LH activity. We have therefore successfully developed a novel expression system that produces functional human LH and enables this normally nonsecretory enzyme to be secreted, facilitating its separation from the intracellular proteins of insect cells. Future applications should allow characterization of the LH active site by crystallographic studies and site-directed mutagenesis for structure-function comparison. ***Key words: Ehlers-Danlos syndrome type VI/hydroxylysines/collagen biosynthesis. J Invest Dermatol 106:11–16, 1996***

Lysyl hydroxylase (LH) is a post-translational modifying enzyme that hydroxylates lysine residues at the Y position of Gly-X-Y peptide sequences in collagen biosynthesis [1]. The resulting hydroxylysines are essential for the formation of the intermolecular cross-links [2] that give collagen its tensile strength. LH exists as an α_2 dimer of two identical 85-kDa subunits, each consisting of 709 amino acid residues and a signal peptide of 18 additional residues [3]. The enzyme requires Fe^{++}, ascorbate, α-ketoglutarate, and oxygen as cofactors for catalytic activity [1]. LH has recently been shown to reside loosely bound to the endoplasmic reticulum *via* weak electrostatic bonds [4]. For this reason, assays of LH activity in human skin fibroblasts, which can measure only solubilized enzyme, have depended on the use of detergents that, above certain concentrations, decrease enzyme activity. Our laboratory routinely measures LH activity in a 0.1% Triton X-100 extract of fibroblast cell homogenate, in which the enzyme appears to retain full activity [5].

The importance of LH is emphasized by the autosomal recessive disorder Ehlers-Danlos syndrome type VI (EDS VI), in which LH deficiency appears to be responsible for the clinical phenotype of joint laxity, kyphoscoliosis, muscle hypotonia, and soft, bruisable skin [6]. Patients with EDS VI are also susceptible to corneal abnormalities [7] and catastrophic arterial rupture [8]. The diagnosis of EDS VI is based on the clinical phenotype and verified by an assay of decreased LH activity in cultured skin fibroblasts. Clinical evidence suggests that LH deficiency may be produced by various types of mutations affecting the LH primary structure, and this has been confirmed recently by the characterization of four different mutations in the cDNAs for LH [9–11].

Baculovirus expression systems are useful tools for the production of recombinant proteins that require eukaryotic post-translational modifications such as glycosylation, disulfide bonding, phosphorylation, and signal sequence cleavage [12]. A baculovirus expression system has been used to express a fully active form of human prolyl 4-hydroxylase (PH), an α_2-β_2 tetramer, in which the β subunit is protein disulfide isomerase (PDI). Both PH and LH require the same cofactors for catalytic activity [13,14], and the enzymes may require glycosylation to be fully active [15]. Recently, the α subunit of PH from *Caenorhabditis elegans* has been coexpressed with the β subunit of human PH (PDI) in insect cells by a baculovirus system. This resulted in the production of an active form of PH that exists as an $\alpha\beta$ dimer [16]. Based on the success of the baculovirus system to express active PH, it seemed likely that

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Abbreviations: EDS VI, Ehlers-Danlos syndrome type VI; LH, lysyl hydroxylase; MOI, multiplicity of infection; PDI, protein disulfide isomerase; PH, prolyl 4-hydroxylase.

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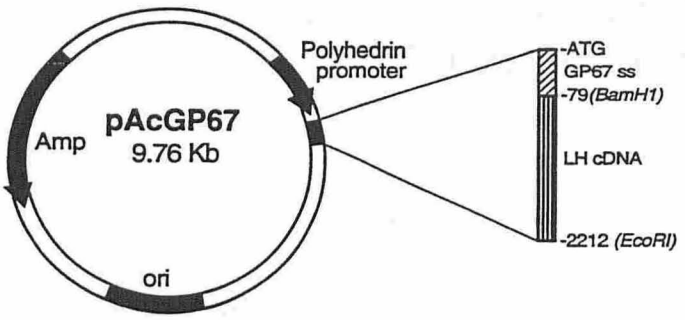

Figure 1. Synthesis of the pAcGP67-LH construct. This construct was synthesized such that the GP67 secretory signal sequence (ss, *diagonal lines*) of the pAcGP67 transfer vector (containing the ATG start site) was ligated in frame with the coding region of human LH cDNA (*vertical lines*), selectively amplified between nucleotides 79 and 2212, so that the GP67 sequence replaced the native signal sequence of LH. *Bam*H1 and *Eco*R1 sites were attached to nucleotides 79 and 2212, respectively, as shown. The ligated sequences were under the direction of the polyhedrin promoter.

this system could be used to express functional human LH. Our initial attempts to express active LH using a construct in which the human LH native signal sequence was retained were unsuccessful, possibly because of problems in solubilizing this membrane-bound enzyme (unpublished data). We therefore used the pAcGP67 transfer vector containing the signal sequence from a baculoviral envelope glycoprotein, GP67, which is essential for the entry of the AcNPV baculovirus into insect cells. In the present study, we synthesized a construct of LH cDNA and pAcGP67, in which the native signal sequence of LH was replaced with the baculoviral GP67 signal sequence, that was able to direct the secretion of LH as a functional enzyme. We thus report the successful expression of active human LH, using a novel system for secretion that greatly facilitates the purification of this normally membrane-bound enzyme.

MATERIALS AND METHODS

Isolation of mRNA and cDNA From Fibroblasts Dermal fibroblasts from a normal donor were seeded at 4×10^5 cells per 10-cm tissue culture dish and grown to confluence in Dulbecco's modified Eagle's medium supplemented with 20% dialyzed heat-inactivated fetal bovine serum, as described previously [17]. Total RNA was extracted from the cultured fibroblasts using the acid-guanidinium thiocyanate–phenol-chloroform extraction method [18]. Poly(A) RNA was isolated from the total RNA extract using a Micro-fast Track Kit (Invitrogen). First-strand cDNA synthesis was primed with an oligo dT adapter (Invitrogen cDNA kit).

Amplification of a Partial LH cDNA A 2133–base-pair LH cDNA (containing the entire coding region with the exception of the signal sequence) was amplified by polymerase chain reaction (PCR) from the fibroblast cDNA pool using a 5′ primer based on the LH cDNA sequence (Genbank accession number M98252) between nucleotides 79* and 97, 5′-G/*GATCCA**AGGGCGACGCCAAGCCGG-3′, with an attached *Bam*HI site (*italics*) and a 3′ primer, complementary to the following sequence between nucleotides 2195 and 2212*, 5′-TCGTCGATCCC-TAATTGG**G/AATTC*-3′, with an attached *Eco*R1 restriction site (*italics*). PCR amplification was carried out for 40 cycles in 50-μl volumes using 10 ng cDNA, 30 ng of each primer, 100 μM of each dNTP, 1.5 mM Mg^{++}, 50 mM KCl, 10 mM dithiothreitol, 10 mM Tris-HCl pH 8.3, 0.01% gelatin, and 1.25 U Taq polymerase (Gibco BRL), with denaturation at 94°C for 1 min and 10 seconds, annealing at 56°C for 2 min and 10 seconds, and extension at 72°C for 6 min.

Preparation of the LH cDNA/pAcGP67 Construct The amplified LH cDNA and the vector pAcGP67 (PharMingen) were restriction digested with both *Bam*H1 and *Eco*R1, and gel purified. The restricted vector was phosphatased with bacterial alkaline phosphatase (Gibco BRL) and directionally ligated in a 2:1 molar ratio of insert to vector so that the signal sequence of the vector was in frame with the LH cDNA under the control of the polyhedrin promoter **(Fig 1)**. After overnight ligation at 14°C, the reaction mixture was transformed into competent *Escherichia coli* DH5αF′ and plated on LB/ampicillin (50 μg/ml) at 37°C. The resulting colonies were suspended in 100 μl LB broth, positive clones identified by PCR analysis were amplified, and the construct DNA was isolated by Qiagen maxi plasmid kits (according to the manufacturer's instructions). The LH cDNA insert was then partially sequenced [19] to confirm that the ligation was correct and was then completely sequenced (University of Florida Interdisciplinary Center for Biotechnology Research Facilities) to determine the accuracy of PCR amplification.

Insect Cell Culture Sf9 insect cells were grown in Sf900 II serum-free media (Gibco BRL) containing 1 × antibiotic/1 × antimycotic (Gibco BRL) at 27°C in spinner flasks at 80 rpm. Cells in log-phase growth were seeded at 5×10^5 cells/ml and split every third day upon reaching 2×10^6 cells/ml.

Preparation and Analysis of Recombinant Baculovirus The construct was cotransfected with AcNPV baculoviral DNA into log-phase Sf9 cells using the Baculogold system, according to the manufacturer's instructions (PharMingen). Viral supernatant was harvested at day 5 and reamplified three times at a multiplicity of infection (MOI) of less than 0.3. Upon achieving a viral titer of approximately 10^8 virus particles/ml, a monoclonal viral stock was grown from an isolated plaque, amplified two times at an MOI of approximately 0.1, and harvested at 72 h. The supernatant was confirmed to contain the LH cDNA construct by a slot-blot assay [20] using a ^{32}P-labeled LH cDNA probe (amplified between nucleotides 79 and 2212) [21]. Plaque assays were performed at different stages of amplification according to the manufacturer's instructions for Baculogold to assess viral titer. Northern blot analysis of cell lysates was also performed as described previously, using a similar LH cDNA probe as in the slot-blot assay [22]. Wild-type AcNPV baculovirus was also amplified by this method and used as a control throughout these experiments. No LH cDNA or LH mRNA could be detected in the wild-type virus.

Production of Recombinant Protein 2×10^7 Sf9 cells in log-phase growth with greater than 98% viability were seeded on 15-cm tissue culture plates containing Sf900 II medium (Gibco BRL). After 6 h, high-titer viral LH (10^8 pfu/ml) or wild-type stock was used to infect separate plates of cells, each at an MOI of approximately 5. After an additional hour, 10 ml of serum-free medium was added to the plates, which were then incubated at 27°C. Cells and supernatant were harvested 44 h later and stored at −70°C if not used immediately. These fractions were prepared for the assay of LH activity, as described in the next section.

Isolation of Enzyme for Activity Assays

Cellular Fraction: This assay was performed as described previously for dermal fibroblasts [5], in which the enzyme is retained in the cell, bound to the endoplasmic reticulum. The infected insect cells were frozen and thawed, sonicated, and solubilized in 0.1% Triton X-100, and the cellular extract was centrifuged for 1 h at 27,000 × *g*. This supernatant, containing any enzyme extracted from the cells, was then assayed for LH activity.

Supernatant Fraction: Because there was an indication that the serum-free medium (Sf900 II) may contain an inhibitor of LH activity,* the supernatant was washed thoroughly before enzyme assay. Twenty milliliters of the supernatant fraction from the infected cells was concentrated twofold with a Centriprep-30 device (Amicon). This was then diluted with 10 ml of 20 mM Tris-HCl, pH 7.5, and reconcentrated by tenfold; the 2-ml concentrate was again diluted to 20 ml with the same buffer. This concentration step, followed by the buffer replacement, was repeated three times, ending with a final sample of 2 ml. The activity of LH in this sample was measured, and it was then used as an enzyme source for further experiments.

LH Activity Assay Each of the extracts to be analyzed was added at 0.6 ml to a reaction mix of 1.5 ml containing 50 mM Tris-HCl at pH 7.8, 0.5 mM α-ketoglutarate (Sigma), 5 mM sodium L-ascorbate, 1 mM dithiothreitol, 0.1 mg/ml catalase (Sigma), and 1×10^6 cpm of dialyzed L-[4,5-^{3}H]lysine-labeled, underhydroxylated collagenous substrate from human fibroblasts, prepared as described previously [23]. To assay for the inhibitory effect of the serum-free medium, 0.5 ml of either buffer or serum-free medium was added to 0.1 ml of identical fibroblast extracts, and activity was measured as described. Enzyme assay reactions were incubated at 30°C in a shaking water bath for 2 h, stopped with 0.1 ml of 50% trichloroacetic acid (Sigma), and then distilled at 70°C under reduced pressure in a custom-

* To date, we have been unable to ascertain the identity of this inhibitor because the contents of the medium are protected by the manufacturer; however, Fe^{++} may be implicated, as excess Fe^{++} has been shown to inhibit LH activity in the recombinant protein.

made glass apparatus. A 1-ml aliquot of the distillate was mixed with 10 ml of Aquasol-2 (DuPont-New England Nuclear) and counted in an Intertechnique liquid scintillation spectrometer. The addition of 5 mM $FeSO_4$ and 1.5 mg/ml bovine serum albumin, used to assay LH activity in dermal fibroblasts [5], was found to be detrimental to the assay of the recombinant enzyme.

Enzyme Kinetic Studies The Km values were determined for α-ketoglutarate and ascorbate by analysis of LH activities using washed supernatant, as described for the LH activity assay, using a variable concentration of the particular cofactor to be analyzed. Double reciprocal plots of LH reaction velocity (y-intercept) versus cofactor concentrations (x-intercept) gave the Km values for α-ketoglutarate using concentrations from 0 to 0.5 mM and for ascorbate using concentrations from 0 to 5 mM, as determined from the negative reciprocal of the x-intercept. LH activity was measured for the volume of procollagen substrate between 0 and 0.4 ml.

Time Course Study for Maximal Protein Production Six 15-cm plates were seeded with 2×10^7 serum-free Sf9 cells, and cells were allowed to attach to the plate. After 6 h, high-titer viral stock (10^8 pfu/ml) was added to the cells at an MOI of approximately 5, and the cells were incubated at 27°C. Supernatant was collected from three plates each at 24, 44, 48, 72, and 96 h after infection; centrifuged to remove cells; and frozen immediately at −70°C if not directly assayed.

Purification of LH Unconcentrated supernatants at 100 ml from LH-recombinant infected Sf9 cells were dialyzed three times in 2 l of buffer containing 20 mM Tris pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% NaN_3 for 8–10 h per buffer change. This dialyzed supernatant was loaded at about two drops/min on a column with a 1-ml square matrix bed of concanavalin A sepharose 4B (Pharmacia) overnight at 4°C, and then washed with a minimum of 20 bed volumes of dialysis buffer. The column was eluted with the same buffer containing a stepwise gradient of methyl-α-D-pyranoside (Sigma) up to a concentration of 1 M. Eluted fractions, 15 μl each, were electrophoresed at 150 V for 1 h on 7.5% polyacrylamide minigels (Biorad). The gels were silver stained per the manufacturer's protocols (Biorad). Fractions identified as containing an 85-kDa protein were assayed for LH activity, pooled, and concentrated by a Centriprep-30 (Amicon) before N-terminal amino acid sequencing.

N-Terminal Amino Acid Sequence Analysis The concentrated fractions containing the 85-kDa protein were separated by sodium dodecylsulfate-7.5% polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then electroblotted onto a polyvinylidene membrane (Immobilon) and stained briefly with Coomassie blue. The 85-kDa protein was cut from the membrane and subjected to automated Edman degradation in an Applied Biosystem 477 A pulse-liquid phase sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystem 120 A high-performance liquid chromatography system. Both instruments were operated as recommended in user bulletins and manuals distributed by the manufacturer [24].

RESULTS

Synthesis and Analysis of the pAcGP67/LH cDNA Construct A 2.1-kb cDNA containing the complete coding region for LH, excluding the native signal sequence, was amplified by PCR from normal human skin fibroblast cDNA. The cDNA was directionally ligated into the pAcGP67 vector **(Fig 1)**, transformed into competent *E. coli* DH5αF′, and plated with ampicillinase selection overnight. PCR analysis showed that each of the six colonies that grew on the plate contained the LH cDNA insert. Initial sequencing of the plasmid DNA isolated from one of the colonies verified that LH cDNA was correctly ligated in frame with the GP67 signal sequence of the vector. The complete sequence of the insert cDNA revealed two base changes from our previously amplified LH cDNA sequence from normal human skin fibroblasts [22], which would produce amino acid changes of K330R and S660T, respectively. These changes, which would not produce any alteration in net charge or hydrophobicity/hydrophilicity of the protein, may be the result of either PCR-based mutations or previously undetected polymorphisms present in the other LH allele amplified from our normal cell strain. In either event, these changes do not appear to make a significant difference to LH activity, as the enzyme activity expressed in insect cells appears to be comparable to the level expressed by a similar number of fibroblast cells (data not shown).

Isolation and Amplification of the Recombinant pAcGP67/LH Baculoviral Supernatant Sf9 insect cells were cotransfected with the LH-pAcGP67 construct together with linearized, deficient AcNPV baculoviral DNA, which allows a recombination event to occur between homologous regions of the construct and the baculoviral DNA such that baculoviral function is rescued and the gene of interest is integrated into the viral genome. Recombinant viral supernatant was amplified and a monoclonal viral stock was grown from an isolated plaque, which was then reamplified. This viral stock was confirmed to contain LH cDNA by slot-blot hybridization, and Northern blot analysis confirmed the presence of a 3.2-kb LH mRNA, the same size as LH mRNA from skin fibroblasts, in the cellular extract (data not shown).

Production of Recombinant Secreted LH After the infection of Sf9 cells with high-titer viral stock to produce recombinant protein as described in *Materials and Methods*, significant LH activity was measured in the supernatant of cells infected with the LH-recombinant virus. In contrast, no activity was detected in the supernatant of Sf9 cells that had been infected with wild-type AcNPV baculovirus. Intracellular fractions isolated from cells infected with either the wild-type or LH-recombinant virus showed no LH activity, demonstrating the efficiency of the GP67 signal sequence to direct the secretion of recombinant LH from Sf9 cells.

LH Activity Is Inhibited by Media Interestingly, in our LH assays we observed a significant inhibition of activity by the Sf900 II medium (Gibco BRL) used in the Sf9 cell culture. The inhibitory property of this medium, which was reversible, affected both active LH expressed by the baculovirus system and the enzyme isolated from human dermal fibroblasts. LH activity was measured (by tritiated H_2O release, as described in *Materials and Methods*) in duplicate fibroblast extracts of LH to which either Sf900 II medium or an equal volume of buffer was added. Addition of Sf900 II reduced the LH activity to 43 cpm above background, in contrast to the control value (buffer alone) of 323 cpm above background; this represents an 87% inhibition of LH activity. A similar inhibition of LH activity was demonstrated using several other commercially available serum-free insect cell media (data not shown). We found that the inhibitor present in Sf900 II could be removed by several rounds of concentrating the supernatant using a membrane that retains particles larger than 30 kDa and then replenishing the retentate volume with buffer, as described in *Materials and Methods*. This washing step produced a 10-fold increase in LH activity, and this enzyme preparation was used in all subsequent assays.

Kinetic Analysis of Recombinant LH Assay of enzyme activity in the supernatant of Sf9 cells infected with the viral recombinant showed a linear increase in activity with volume of supernatant. This activity was completely inhibited by the addition of α,α′-dipyridyl (5 mM), which chelates Fe^{++}, one of the cofactors required for LH activity. To determine the kinetics of the recombinant enzyme for two other cofactors required for LH activity, α-ketoglutarate and ascorbate, we varied the concentration of that cofactor under otherwise constant conditions and assayed the LH activity. The enzyme displayed first-order kinetics for both cofactors from which the Km values could be determined. As shown in **Table I**, these values fall into the range of previously published Km values for LH from chick embryo and LH from human placenta and dermal fibroblasts [25–28]. The recombinant enzyme also displayed first-order kinetics for the procollagen substrate.

Verification of Identity of Expressed Protein by Size, Activity, and Sequence Analysis In a time course study, Sf9 cells were infected with high-titer recombinant viral stock for several different incubation times over a 96-h period. LH activity assays showed maximum accumulation of enzyme at 44 h **(Fig 2)**. After this time, it appeared that the rate of degradation or deactivation of LH exceeded the rate of active LH production. This 44-h point was used for the subsequent isolation of supernatant to provide sufficient LH for purification and analysis. The enzyme was purified using a concanavalin A sepharose column, which binds glycosylated proteins [29]. Eluted fractions were separated by SDS-PAGE, and silver staining of the gel revealed a prominent band in several

Table I. Comparison of Km Values for Ascorbate and α-Ketoglutarate of Recombinant LH With Previously Published Values for Native LH Isolated From Human and Chick[a]

Source of LH	Cofactors (μM)	
	Ascorbate	α-Ketoglutarate
Recombinant[b]	40	70
Human fibroblasts [25][c]	4	20
Human fibroblasts [26][c]	100	
Human placenta [27][c]	200–400	40
Chick embryo [28][c]	220	70

[a] Km values were obtained for baculovirus-expressed LH and the cofactors ascorbate and α-ketoglutarate by Lineweaver-Burke plots of LH reaction rate versus concentration of each cofactor. These values are compared with published Km values for native LH isolated from sources as shown. The wide range of published values is presumably due to the degree of enzyme purity and the effect of detergent use on activity.

[b] Assay performed at 30°C and pH 7.5.

[c] These assays were performed at 30°C to 37°C and between pH 7.2 and 7.8.

fractions corresponding to an 85-kDa protein, the expected size for LH **(Fig 3*A*)**. The co-elution of a second band at 30 kDa (data not shown) has been observed previously from columns using this matrix [30] and is presumably concanavalin A. The intensity of the 85-kDa band correlated with the LH activities measured in the same fractions **(Fig 3*B*)**. The total yield of purified LH was estimated as between 1 and 10 mg/l of supernatant.

In preparation for sequence analysis, the enzyme purified from concanavalin A sepharose was electrophoresed by SDS-PAGE and transferred to a polyvinylidene membrane [24], followed by brief staining with Coomassie blue to locate the 85-kDa protein. N-terminal amino acid sequencing of the excised protein showed that the protein was human LH and that the signal sequence had been removed correctly **(Table II)**.

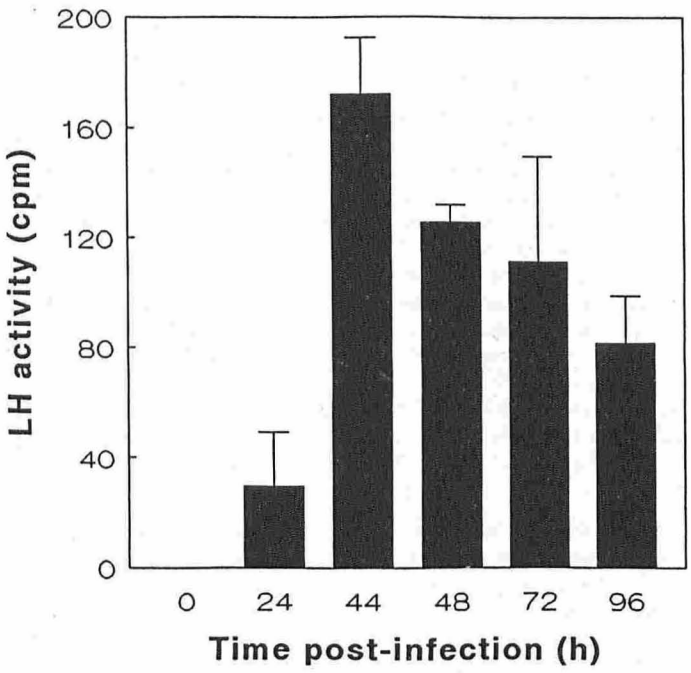

Figure 2. LH is produced maximally at 44 h during a 96-h viral infection of Sf9 cells. LH activity (expressed in cpm ^3H_2O/min less background) was measured in the supernatant of Sf9 cells infected with high-titer recombinant viral stock for 24, 44, 48, 72, and 96 h, as described in *Materials and Methods*. The results from three separate experiments are plotted as mean LH activity ± SD versus time post-infection.

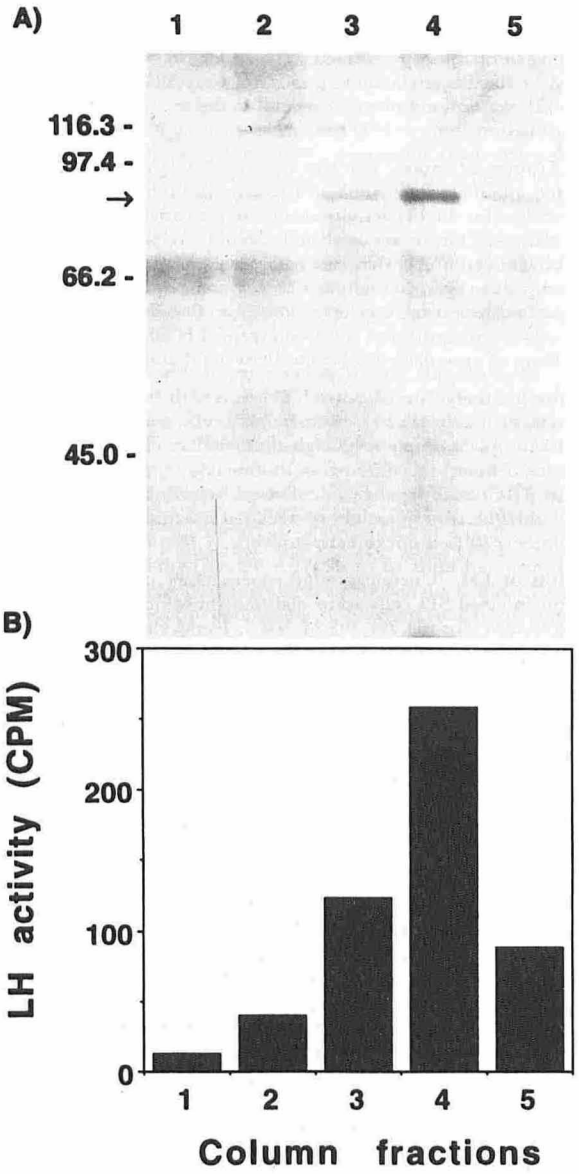

Figure 3. Correlation of LH activity with the concentration of an 85-kDa protein present in fractions containing recombinant proteins purified by concanavalin A Sepharose chromatography. Sf9 cells were infected with high-titer viral stock for 44 h to produce recombinant protein, as described in *Materials and Methods*. The enzyme was purified from the supernatant using a concanavalin A sepharose column. *A*, the column fractions (*lanes 1–5*) were eluted with methyl-α-D-pyranoside (0.3–0.4 M) and analyzed by SDS-PAGE followed by silver staining, which identified a major protein of 85 kDa (*arrow*). Molecular-weight markers are indicated to the left of the gel. *B*, LH activity (expressed in cpm ^3H_2O/min less background) was assayed in each of these fractions as described in *Materials and Methods* and correlated exactly with the concentration of the 85-kDa protein, as indicated by band intensity. Duplicate experiments showed that these assay results were reproducible to within 10%.

DISCUSSION

In this study, we report the successful expression of a functional, secreted form of human LH, a protein normally bound to endoplasmic reticulum, using a baculovirus expression system. To accomplish this, we cloned an LH cDNA into the multiple cloning site of the transfer vector pAcGP67 such that the native signal sequence of LH was replaced by a powerful signal sequence for secretion. Cotransfection of the pAcGP67-LH cDNA construct and AcNPV baculoviral DNA into Sf9 insect cells produced a recombinant virus with which we were able not only to express an active

Table II. N-Terminal Amino Acid Sequence of Recombinant 85-kDa Protein Shows Identity With Native LH[a]

GP67 Signal Sequence	Linker	Human LH (Source)
		KGDAKPEDNLLVLTV (native)
	AXLGS	**XGDAKXEDNLLVLTV (recombinant)**
MVSAIVLYVLLAAAAHSAFA	ADLGS	
	↑	

[a] The 85-kDa band excised from an SDS-PAGE gel was analyzed by automated Edman degradation, as described in *Materials and Methods*. *First line* (*bold*), published N-terminal amino acid sequence for human LH (native) between nucleotides 79 and 93 [3]. *Second line* (*bold*), N-terminal amino acid sequence of the excised 85-kDa protein (recombinant), which is identical to human LH apart from two residues (and one residue in linker sequence) that could not be clearly identified, designated by an "X." The five-residue linker sequence, to ensure correct cleavage of the signal sequence, remains attached to the N-terminus of the recombinant LH. *Third line*, GP67 signal sequence (*italics*) including the linker of five residues cleaved at site shown (*arrow*).

form of human LH, but also to express this intracellular enzyme in a secreted, easily purifiable form.

Earlier attempts in our laboratory to express functional LH in a baculovirus system, using a construct (LH-pVL1392) in which the native signal sequence of LH was intact, were unsuccessful (M. Marshall and H. Yeowell, unpublished data). In these attempts, hybridization analysis showed that the synthesis of both DNA and mRNA for LH had occurred; however, no LH activity could be detected in either cellular extracts or supernatants from the infected cells. In human dermal fibroblasts, in which LH is retained loosely bound to the endoplasmic reticulum [4], we were able to isolate LH in its fully active form in a 0.1% Triton X-100 extract of cell homogenate [5]. In insect cells, however, even the use of up to 1% concentrations of Triton X-100 or SDS failed to release an active form of LH. Because our activity assays depend on the availability of soluble LH, our inability to detect LH activity could have been due to failure of the expressed enzyme to be solubilized under conditions in which activity is retained.

Interestingly, although the native form of PH, an $\alpha_2\beta_2$ tetramer, was expressed as a functional enzyme when both subunits were cotransfected into a baculovirus expression system, the individual α- and β-subunits were differentially retained when expressed individually in insect cells [13]. The β-subunit of PH was expressed in its fully active form as PDI after extraction with 0.1% Triton X-100, whereas only traces of the α-subunit of PH, in either of its spliced forms, were detectable under similar extraction conditions. This is somewhat analogous to our initial attempt to express functional LH, because the α-subunit of neither PH nor LH has either of the signals for protein retention in the endoplasmic reticulum (KDEL or a double lysine motif), although both proteins reside in the lumen of the endoplasmic reticulum [4]. In addition, it has been reported recently that LH co-localizes loosely bound to PDI, the β-subunit of PH, and other KDEL-containing proteins in the endoplasmic reticulum [4]. However, whereas the α-subunit of PH requires PDI to maintain its catalytic activity, LH appears to require only one type of subunit for its activity, a requirement that we have confirmed in this present study.

Because of our initial problems with isolating a functional enzyme that normally exists in a membrane-bound form, in the present study we designed a construct in which the native signal sequence of LH was replaced with a GP67 baculoviral signal sequence for secretion, which was able to direct the secretion of LH. This powerful signal sequence of GP67, an acidic glycoprotein that is essential for the entry of baculovirus into insect cells, has been used successfully to direct the secretion of the Leishmania surface metalloproteinase GP63 in a functional, glycosylated form [30]. The supernatant of Sf9 insect cells infected with the viral recombinant, into which the LH cDNA-pAcGP67 construct had been incorporated, showed LH activity that increased linearly with increasing volume of active supernatant; this effect would be expected under conditions of saturating concentrations of substrate and cofactors. The absence of LH activity in the infected cell lysate suggested that the GP67 signal sequence was very effective in directing the secretion of the recombinant enzyme. Neither cDNA, mRNA, nor activity of LH could be detected in the supernatant or cell lysate from insect cells infected with the wild-type AcNPV baculovirus. It is interesting that although these insect cells lack any detectable LH, we have observed PH activity in both the cell lysate and supernatant of insect cells (unpublished data). This suggests that the processing of collagen, which normally requires the action of both LH and PH, occurs differently in insect cells.

In this study, we have shown that the recombinant enzyme requires three cofactors: Fe^{++}, α-ketoglutarate, and ascorbate [2]. The addition of α,α'-dipyridyl, which chelates Fe^{++}, completely inhibited enzyme activity in the recombinant supernatant. Moreover, the enzyme demonstrated first-order kinetics for α-ketoglutarate and ascorbate in reactions from which the Km values could be calculated. These values were in the same range as those previously published for LH from chick embryo [28], human skin fibroblasts [25,27], and human placenta [28], indicating that the kinetic properties of this recombinant enzyme are similar to those of the native enzyme. In addition, the expressed LH demonstrated first-order kinetics for the underhydroxylated procollagen substrate derived from human fibroblasts; however, this biologic substrate could not be sufficiently quantitated to provide an accurate Km value.

It has been reported that glycosylation of LH is required for the enzyme to be active [15]. The presence of active LH in the supernatant from the infected cells suggested that the enzyme was glycosylated and therefore could be purified by concanavalin A sepharose chromatography, which binds glycosylated proteins. SDS-PAGE analysis of fractions eluted from a concanavalin A sepharose column revealed a prominent band corresponding to an 85-kDa protein, the expected size of the LH subunit [28]. Further evidence that this protein was LH was provided by the close correlation between LH activity and the intensity of the 85-kDa band of these fractions. Analysis of the combined fractions containing purified enzyme suggested that between 1 and 10 mg of concanavalin-A–purified LH could be isolated from 1 l of supernatant. N-terminal amino acid sequencing of the 85-kDa protein excised from the gel showed that all 13 identifiable amino acids were identical to human LH; the identity of the two other residues could not be confirmed. The presence of the first five residues, included in the pAcGP67 vector to ensure that the signal sequence was correctly cleaved, provided further evidence that the expressed LH was from our viral construct and not an endogenous protein from insect cells.

LH has been an extremely difficult protein to purify by conventional methods [15], and very little is known about its structure and active site. The development of this expression system, which allows secretion of a functional form of LH, provides an excellent source of pure LH for x-ray crystallography, from which the structure and active site of LH can be characterized. This study can be complemented by using site-directed mutagenesis [31] to mutate regions of the gene identified as important for activity both by analysis of mutations characterized in the LH cDNAs from EDS VI patients, and by the identification of sequence conservation across species. The functional site(s) can then be defined by assessing the changes in activity caused by various mutations introduced into the gene. Currently, four mutations have been identified in the cDNAs for LH in fibroblasts from patients with EDS VI [9–11]. In one of these cases, the patient was shown to be a compound heterozygote

for the LH gene with a significant mutation in each allele of E532 del and G678A, respectively [9]. The second mutation of G678A disrupts the 97% conserved C-terminal region identified in human and chick LH between residues 620 and 708, in which two histidine motifs are thought to be necessary for LH activity. These data suggest that the C-terminal region provides an ideal target for site-directed mutagenesis.

We thus report the successful development of a baculovirus system that expresses functional human LH, as evidenced by amino acid sequence, protein size, enzyme activity, and enzyme kinetic studies. In addition, this system secretes this normally nonsecretory enzyme into media, thus simplifying the purification of LH from intracellular proteins of insect cells. Future applications of this system should allow characterization of the functional domain(s) of LH by x-ray crystallographic studies and site-directed mutagenesis followed by structure/function analysis. Furthermore, an understanding of the LH active site should facilitate pharmacologic manipulation of LH activity in the treatment of fibrotic disorders and diseases related to decreased LH activity (EDS VI).

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